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Serotyping of *Clostridium difficile*

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A total of 246 live *Clostridium difficile* cultures were serotyped by a slide agglutination technique. Fifteen grouping antisera were produced which serotyped 98% of the cultures (241 of 246). Our results indicated that certain serogroups may have specific pathogenicity. Strains of serogroups A, G, H, K, S1, and S4 were cytotoxigenic and were isolated mainly from adult patients with pseudomembranous colitis or antibiotic-associated diarrhea. Nontoxigenic strains of serogroups D and Cd-5 were isolated mainly from asymptomatic neonates and small children. Some cross-reactions occurred among some strains of serogroups A, Cd-5, G, and K. These strains were further examined by analysis of protein profiles and restriction endonuclease patterns to elucidate their serology. Typing of *C. difficile* by using slide agglutination is a simple technique suitable for routine examination. Serogrouping may be a useful epidemiological marker and could help in elucidating the medical relevance of some *C. difficile* isolates.

Clostridium difficile is a major etiological agent of pseudomembranous colitis (PMC) and is often isolated from patients with antibiotic- or chemotherapeutic-associated diarrhea (1, 12, 13). Clusters of cases or outbreaks of *C. difficile* as nosocomial infections have been described (5, 8, 15). The carriage rate of *C. difficile* in asymptomatic adults ranges from 0 to 11% (6, 14), but in young children, particularly in neonates, the carriage rate could be as high as 50 to 75% (9–11).

To better understand the pathogenicity of *C. difficile* and its epidemiology, various typing systems have been described. Two methods, serogrouping by slide agglutination (3) and polyacrylamide gel electrophoresis (PAGE) (4, 16), are accepted practical methods in routine typing, as applied to clinical studies (2, 7). The purpose of this study was to assess the slide agglutination technique for typing *C. difficile* and the use of PAGE as a backup and confirmatory technique, when required. Later, we had the opportunity to refer some of our cultures for examination by restriction endonuclease analysis (REA).

MATERIALS AND METHODS

Preparation of immune sera. Eight reference strains of *C. difficile* serogroups A, B, C, D, F, G, H, and X were received from M. Delmée in June 1985. Their ATCC numbers are as follows: A, ATCC 43594; B, ATCC 43593; C, ATCC 43596; D, ATCC 43597; F, ATCC 43598; G, ATCC 43599; H, ATCC 43600; and X, ATCC 43603. They were used as Formol-treated bacterial cells to immunize rabbits by four intravenous injections by the method of Delmée et al. (3). Ten other untypable *C. difficile* strains, isolated from clinical specimens, were used to prepare immune sera.

Slide agglutination technique. The slide agglutination technique of Delmée et al. (3), with formolized cultures, was slightly modified. Live *C. difficile* cultures, incubated overnight on prereduced blood-enriched Columbia agar medium in GasPak jars (BBL Microbiology Systems, Cockeysville, Md.), were used. The best results were obtained by working with 24-h cultures within 4 h after removal from the incubator. Cultures incubated for 48 h produced better growth, but yielded more cross-reactions.

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Bacterial isolates. A total of 246 *C. difficile* strains isolated from clinical specimens was examined. Of these, 168 were from patients with pseudomembranous colitis (PMC), antibiotic-associated diarrhea (AAD), or other, unspecified, gastrointestinal disorders. Other strains were isolated during investigation of an outbreak of acute gastroenteritis in a day care center for small children ($n = 7$), from neonates ($n = 34$), and from patients with various clinical conditions from a chronic renal unit of a large hospital ($n = 44$). Each isolate was identified by its morphological and biochemical characteristics and by gas-liquid chromatographic analysis of its fermentation products. Sorbitol fermentation was determined by the method of Delmée et al. (3).

Cytotoxin assay. Tenfold dilutions of broth culture supernatant were added to Vero and McCoy cell monolayers. *C. sordellii* antitoxin was added, and the mixture was incubated overnight under CO_2. Monolayers were then examined for cytopathic changes. A strain was considered toxigenic when a cytopathic change (a rounding off of the cells) occurred in 50% of the cells.

PAGE. PAGE analysis of whole-cell extracts was carried out for 81 *C. difficile* strains (4).

REA. Fifty-seven *C. difficile* strains were referred to H. Roslyn Devlin, Toronto General Hospital, for REA analysis (H. R. Devlin, W. Au, and W. C. Bradbury, Program Abstr. 26th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 506, 1986) to clarify some of our unresolved serogrouping results.

RESULTS

Using the initial eight antisera, we classified 80% of the isolates into serogroups A, C, D, G, H, and X. None reacted with antisera to serogroups B and F. Immunization of rabbits with the 10 additional *C. difficile* strains yielded antisera for seven new *C. difficile* serogroups. Of these new serogroups, two had been previously designated by Delmée et al. as serogroups I (ATCC 43601) and K (ATCC 43602) (4). The remaining five were designated by us as Cd-5, S1, S2, S3, and S4. Hence, we recognized 15 *C. difficile* serogroups, with which we were able to serogroup 98% of the strains.

Agglutinating titers for all serogroups were 1:200 to 1:400, and the sera were diluted 1:100 to 1:200 for use in the slide agglutination technique. Numerous cross-reactions were b-

TABLE 1. Serogrouping, toxigenicity testing, and sorbitol fermentation of 246 *C. difficile* cultures

Serogroup	No. (%) of cultures[a]	No. (%) of toxigenic cultures		No. (%) of nontoxigenic cultures	
		Total	Sorbitol positive	Total	Sorbitol positive
A	77 (31)	74 (96)	59 (80)	3	2
Cd-5	37 (15)			37 (100)	37 (100)
G	33 (13)	32 (97)	32 (100)	1	1
H	29 (12)	29 (100)	29 (100)		
D	16 (6)			16 (100)	
K	14 (6)	12 (86)	12 (100)	2	
S1	14 (6)	13 (93)		1	
S4	10 (4)	10 (100)	10 (100)		
I	5 (2)			5 (100)	5 (100)
C	2	2	2		
S3	2	2	1		
X	1			1	
S2	1	1	1		
Nongroupable	5 (2)	2	2	3	1
Total	246	177 (72)	148 (84)	69 (28)	46 (67)

[a] No serogroup B or F strains were found.

served, some of which were eliminated by working with cultures as soon as possible after removal from the incubator. The persistence of cross-reactions, particularly for serogroups A, K, G, and Cd-5, meant that additional confirmation by PAGE and REA was required.

The prevalence of *C. difficile* serogroups is presented in Table 1. A correlation was found to exist between serotypes and cytotoxin production and between serotypes and sorbitol fermentation. Two serotypes, Cd-5 and I, bore the unusual feature, for nontoxigenic strains, of being sorbitol positive.

Nearly all strains which belonged to serogroup A were toxigenic (96%) and were isolated from adults with PMC or AAD. Serogroup A was found to be a heterogenous group, displaying cross-reactions with serogroups G, K, and Cd-5. Delmée has described 11 different PAGE profiles within serogroup A, and we found 8 within the same group. Serogroup G strains (13% of all cultures examined) cross-reacted strongly with serogroup A. To obtain final typing results for this serogroup, the PAGE profile was determined and found to be typical and unique for serogroup G. A total of 97% of serogroup G strains were toxigenic and sorbitol positive. The majority of these were isolated from adults with PMC or AAD (a few lacked clinical information). Serogroup H (100% toxigenic) and K (86% toxigenic) strains were isolated mainly from adults with PMC, AAD, or other gastrointestinal disorders; one nontoxigenic serogroup K strain was isolated from a child, and one toxigenic serogroup H strain was isolated from a 9-month-old infant with acute gastroenteritis. Fourteen *C. difficile* strains belonged to the new serogroup, S1. They had a unique PAGE and REA profile, 93% were toxigenic, and all were sorbitol negative. Nine cultures were isolated from adults with various gastrointestinal conditions, two were isolated from patients with nosocomial infections, and three were isolated from neonates. Ten *C. difficile* cultures were serogrouped as S4; they cross-reacted with serogroup S3, but the PAGE profile could differentiate them.

All serogroup Cd-5 and D strains were nontoxigenic and were isolated from neonates or small children. The serogroup I strains, although nontoxigenic in vitro, were isolated from adults with symptoms of gastroenteritis. Very few isolates belonged to serogroups C, S3, X, and S2. Seven strains isolated from small children during an investigation of an outbreak in a day care center belonged to four different serogroups, i.e., serogroups D (four), A (one), Cd-5 (one), and H (one), with only serogroups A and H being toxigenic.

DISCUSSION

There is a need for a method of typing *C. difficile* isolates to better understand the medical and epidemiologic relevance of these isolates, particularly in neonates and young children. An epidemiologic marker for tracing the mode of transmission of this bacterium is also needed.

The slide agglutination technique described in this report, which involves the use of live antigens, is a simple, practical, and rapid method for typing *C. difficile* isolates. Typing by PAGE correlated well with the serogrouping results obtained and complemented serogrouping results for some serogroup A, Cd-5, G, and K isolates which yielded cross-reactions. PAGE was very useful for interpreting and analyzing serogrouping results. For example, within serogroup A, different PAGE profiles were observed, and serogroups G, K and Cd-5 cross-reacted with serogroup A. PAGE and REA, although excellent techniques for *C. difficile* typing, are complex techniques and are available mainly in reference laboratories.

The correlation between serogroups and toxigenicity was clear cut. Strains isolated from clinical cases of PMC and AAD belonged to toxigenic serogroups; i.e., A (96%), G (97%). H (100%), K (86%), and S1 (93%). Our data on serogroup G differed from the previous findings of Delmée et al. (3), who found that members of this serogroup were isolated predominantly from neonates and children; we found serogroup G strains more often in symptomatic adults. Serogroup Cd-5 and D strains were all nontoxigenic and were isolated from asymptomatic neonates and small children.

During this study we applied this serotyping technique to related strains isolated during an outbreak of gastroenteritis in a day care center. Four different serogroups were found among the seven *C. difficile* strains isolated, which ruled out *C. difficile* etiology. Viral etiology was later established.

Our results confirm previous observations of Delmée et al. that *C. difficile* strains of different serogroups have different pathogenic properties. All toxigenic serogroups (A, G, H, K, and S1) were isolated most frequently in symptomatic

adults; nontoxigenic serogroups such as Cd-5 and D were found predominantly in asympt matic neonates and y ung children. Serogroup I requires further assessment in this regard. From a practical viewpoint, the isolation of a non-t xigenic *C. difficile* strain could rule ut its pathogenicity.

In retrospect, it is n w clear to us why this bacterial species is known as "the difficult *Clostridium*." Unusual features in the growth of cultures, low-titer antisera, and cross-reactivity among serogroups, aii reinforced the early observations by researchers working with this bacterium . . . "C'est difficile!"

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